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PRINCIPAL INVESTIGATOR(S): Paul Bates, Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

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This work describes the development of a novel system for efficient and rapid introduction of genes into specific tissues or cells in mice i.e. *in vivo* targeted gene expression. Detailed analysis of gene function often relies upon introduction of genes into an animal model system. We are developing a retroviral-based system in which controlled expression of the cellular receptor for the virus in a defined pattern determines the site(s) of infection. Using this retroviral gene delivery system, a gene can be introduced not only in a spatial-specific pattern but also in a temporally-controlled manner. For these studies we will express the virus receptor in mammary cells in transgenic mice using a MMTV LTR. The ability to selectively target retroviral infection has numerous applications in areas as diverse as developmental biology, gene therapy and oncogenesis. Here we will attempt to utilize this system for targeted expression of genes in mammary cells in an animal model.

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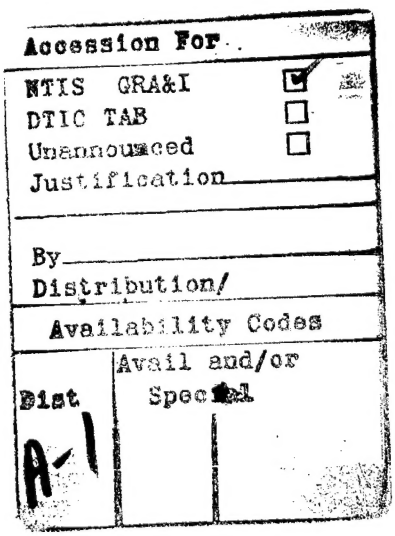

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INTRODUCTION

Presently, the most common way to analyze gene function in a particular cell type *in vivo* is to generate a new transgenic line for each gene under study - a costly and time consuming endeavor. Here we describe an approach which utilizes mice expressing a retroviral receptor transgene (the Rous sarcoma virus receptor) to target infection of retroviral vectors *in vivo*. This allows directed infection, and thus directed gene expression, of cells expressing the viral receptor and provides a rapid and efficient method to test the mammary tumorigenic potential of genes in an animal model. An important difference between this approach and testing gene function in transgenic mice is that infection, and thus gene expression, can be temporally controlled allowing assessment of differences in oncogenic potential at different stages of mammary gland development. Finally, multiple oncogenes can be introduced by co-infection, allowing questions of synergy to be addressed.

The goals of this proposal are: 1) characterize the expression Tva in the mammary gland and 2) optimize Tva-directed infection of mammary cells *in vivo* using vectors carrying histochemical marker genes. Importantly, we have already proven that targeted infection can be accomplished *in vivo*. Efficient infection of myoblasts by avian retroviral vectors was shown in transgenic mice expressing this receptor in muscle. We have also shown that this receptor functions to mediate infection of mammary epithelial cells in culture. Further, we have generated transgenic mice carrying the viral receptor under control of a mammary-specific promoter, the MMTV LTR. Thus, we are poised to continue development of this system for our studies of gene function and oncogene cooperation during mammary tumorigenesis.

1. Isolation and characterization of the subgroup A RSV receptor.

A gene transfer strategy was used to isolate a chicken gene encoding the subgroup A RSV receptor, *tva* [1, 2]. Mammalian cells are normally completely refractory to RSV-A infection. However, the *tva* gene renders mammalian cells as susceptible to RSV-A infection as avian cells [1, 2]. We have introduced the receptor gene into numerous different mammalian cell lines in species ranging from mouse to monkey, and in all cases Tva efficiently induces susceptibility to RSV-A. Taken together these results suggest that Tva can function to mediate efficient infection of many, if not all, mammalian cells.

2. Transgenic mice with a-actin promoter/tva: muscle specific receptor expression.

In collaboration with Steve Hughes at NCI we produced mice which carry RSV receptor transgenes (see attached PNAS paper,[3]). Using a muscle specific alpha-actin promoter/tva construct, we established five mouse lines carrying this transgene. Characterization of these lines by Western blot analysis demonstrates that the receptor is specifically expressed in several types of muscle. Although the level and pattern of expression in muscle vary for each of the transgenic lines, these experiments demonstrate that in general, the receptor can efficiently expressed without deleterious effects. Furthermore, using mice carrying a ß-actin promoter/tva construct we have demonstrated expression of Tva in numerous cell types including early embryonic cells further suggesting that expression of Tva in most contexts is not detrimental.

3. RSV infection of myoblasts *in vivo*

Preliminary infection studies have been performed with the alpha-actin promoter/tva transgenic lines [3]. 2000-5000 infectious units of an RSV vector carrying the bacterial alkaline phosphatase gene (RCAS(A)-BAP) were injected IM into 5 day old

mice. At d5 there is significant myogenesis occurring such that the myoblasts, if susceptible to RSV-A, should be good targets for infection. Controls for the experiments included injection of a subgroup E RCAS-BAP vector which should not utilize the subgroup A receptor and injection of non transgenic littermates. As expected, infection was seen only when the subgroup A virus was injected into transgenic mice. Several hundred infected myoblasts or myotubes are spread throughout the muscle and infection did not seem to be localized at the injection site. Furthermore, by injecting avian cells expressing the RSV vectors rather than the virus stock, infection of the myoblasts was dramatically increased such that thousands of cells appear to be infected. These experiments provide proof of principal for the use of Tva to efficiently target cells for RSV-A infection *in vivo.*

BODY

In our statement of work for this project as task 1 we propose to construct a transgene which will give exppression of the RSV viral receptor in mammary cells and generate transgenic mice carrying this gene. We have accomplished this task. Two of the goals of task 3 have also been addressed. We have constructed the retroviral vectors with histochemical markers required for testing targeting in vivo. Finally, preliminary infection experiments with these vectors have been initiated.

A. Expression of Tva in a mammary epithelial cell line allows RSV infection.

Although we have demonstrated that Tva functions in a number of cultured cells and can infect muscle cells *in vivo* we wanted to test whether mammary epithelial cells would utilize the receptor to allow RSV entry. To address this question, and to determine if the plasmid to be used as the transgene was functional, an MMTV LTR/*tva* construct was introduced into a cultured mammary epithelial cell line, C57MG. The transfected cells were then challenged with an RSV vector carrying a ß-galactosidase marker. Expression of the receptor renders C57MG cells highly susceptible to RSV infection as judged by ß-gal staining. These experiments demonstrate that at least in cultured mammary cells there is no block to RSV infection if the receptor is expressed, and they suggest that *in vivo* RSV vectors should infect the mammary epithelial cells of MMTV LTR/tva transgenics.

B. Generation of mice with an MMTV LTR/RSV receptor transgene.

The expression pattern of the MMTV LTR in mice has been thoroughly characterized. MMTV LTR constructs have been used extensively to construct transgenic mice for the purpose of expressing genes in the mammary gland [4-8]. Abundant expression in mammary epithelial cells is seen when this promoter is utilized. In addition to mammary gland expression, the MMTV LTR also promotes relatively high levels of expression of transgenes in the salivary gland and the testis. Indeed, in transgenics in which an oncogene is driven by this LTR both mammary and, less frequently, salivary gland tumors are induced. For these experiments, we used a construct with the MMTV LTR in the same orientation immediately upstream of the RSV receptor processed gene. Based on pervious studies using an identical arrangement of the LTR and transgene this construct should promote high levels of receptor expression.

Two transgenic lines of mice carrying the MMTV/tva transgene have been established. Presently, in addition to the infection experiments which form the heart of this short proposal, we are breeding these mice into a C57/B6 background. Unfortunately, the particular cross used to produce the transgenic mice at the University of Pennsylvania transgenic facility has a high spontaneous mammary tumor rate.

Therefore, in preparation for use of these mice in future tumorigenesis studies, we are breeding into a C57/B6 backgroung. The C57/B6 line of mice has a low spontaneous mammary tumor incidence. After the Bl/6 background is established (5-6 generations of backcrossing), then mice homozygous for the transgene will be produced (assuming the transgene does not disrupt an essential gene). Producing homozygotes will simplify maintenance of the lines and obviate the need to screen mice for the transgene before conducting expression or infection experiments.

C. Virus vectors and stocks

MLV(RSV) psuedotypes have been produced using a transient transfection protocol similar to one previously utilized to generate high titer MLV psuedotypes [9-12]. Using human 293T cells and a transient transfection protocol we routinely obtain titers of MLV(RSV) psuedotypes of roughly 10^5 infectious units/ml. Presently, we are optimizing a virus concentration protocol for RSV-A which allows 100-fold concentration of virus stock [13, 14]. We have constructed vectors which express either alkaline phosphatase or a nuclear localized lacZ for use in these studies. This transient virus expression system should allow rapid production of high titer MLV(RSV) vector stocks for use in the infection studies. As a control, we also will use subgroup C RSV psuedotypes. Viruses with this envelope do not use the subgroup A receptor and should not infect the transgenic mice. Positive controls will include viruses carrying the MLV envelope protein and VSV G glycoprotein [12-14]. Having these viruses in hand we are now ready to begin testing the in vivo targeting of mammary epithelial cells.

D. Infection of mammary gland cells *in vivo* and primary cultures *in vitro*.

In preliminary injection experiments we have not yet seen evidence for infection by the RSV psuedotypes. Therefore, while proceeding with the in vivo infections, experiments on explanted primary mammary cells from the transgenic mice will be initiated. Although not in the original plan, these experiments will help clarify if the problems encountered thus far with the infections are technical (due to our technique used for the infections) or biological (that the transgene is not conferring susceptibility as expected). The primary cell cultures will allow us to easily address this question. Also, we can analyze the expression of the receptor protein much more easily in the cultured cells, checking not only for the presence of the receptor but also assaying whether it localizes to the cell surface.

CONCLUSIONS

Although this project is still in its early stages we have made significant progress this year. We have made the transgene constructs, used them to produce and establish two lines carrying the MMTV LTR/RSV transgene, produced virus vectors required for this project and established protocols for production of high titer stocks of viruses carrying these vectors, and finally begun preliminary infection experiments. From this work we can conclude that the transgene is not toxic in vivo and mice can be produced. Also we now know that the RSV psuedotype system will allow rapid and efficient production of virus stocks carrying MLV genomes. This will allow us to utilize the many oncogene constructs already in MLV vectors for our future work on this project.

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